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The Complexity of Enzymic Control of Hydrogen Peroxide Concentration May Affect the Regeneration Potential of Plant Protoplasts[1]

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Total peroxidase, NADH-peroxidase, ascorbate peroxidase, superoxide dismutase, and catalase activities were measured in tobacco (*Nicotiana tabacum*) leaves and in regenerating and nonregenerating protoplasts isolated from the same tissue and cultured for 2 weeks. The specific ranges of H_2O_2 concentration at which the enzymes scavenging the active forms of oxygen may efficiently operate and the activities of those enzymes were determined in an extract from tobacco leaves and in dividing and nondividing tobacco mesophyll protoplasts. The overall H_2O_2-scavenging enzyme activities were similar in both protoplast populations during the 2 to 3 d of culture. After 3 d, the regenerating protoplasts started to divide and both the antioxidant enzyme activities and the total peroxidase activity increased; in contrast, the viability and the H_2O_2-scavenging enzyme activities in nonregenerating protoplasts dramatically decreased. Surprisingly, the regenerative potentiality in dividing protoplasts was specifically correlated with a higher NADH-peroxidase activity, which resulted in a net H_2O_2 accumulation in the cells. Light, which causes the accumulation of active forms of oxygen in photosynthetic organelles, also stimulated catalase and ascorbate peroxidase activities in dividing protoplasts. We suggest that the localization of H_2O_2 rather than its absolute concentration might be responsible for oxidative stress and that controlled amounts of H_2O_2 are necessary to allow proper cell-wall reconstitution and the consequent cell division.

The complex metabolic modifications that protoplasts undergo during their isolation may include an alteration in the oxygen balance, with an overproduction of active forms of oxygen, namely superoxide anion, H_2O_2, and hydroxyl radical (Roubelakis-Angelakis, 1993). Toxic oxygen may affect the morphogenic response of cells and other plant explants (Benson and Roubelakis-Angelakis, 1993, 1994) and its increasing accumulation after protoplast isolation has been indicated as a cause of recalcitrance (Siminis et al., 1994). Cell-wall reconstitution is another crucial step in the regeneration process of protoplasts (Hahne and Hoffmann, 1984; Katsirdakis and Roubelakis-Angelakis, 1992a) and it relies on H_2O_2-dependent POX activity (Mäder et al., 1980). If the ability to scavenge the active forms of oxygen and a high cell-wall reconstitution rate are together necessary for cell proliferation, the control of the H_2O_2 concentration can be of special importance.

Living organisms display a wide range of antioxidant strategies. In higher plants, in addition to a direct radical-scavenging activity performed by chemical species such as ascorbate, α-tocopherol, glutathione, and polyamines (Kumar and Knowles, 1993), a chain of enzymic reactions cooperate in the removal of the active forms of oxygen (Foyer and Halliwell, 1976; Asada, 1992). Superoxide anion is the first harmful reactant generated during oxidative stress and it is dismutated by SOD, different isoforms of which are specifically localized in the cytoplasm, mitochondria, and chloroplasts (Bannister et al., 1987). The resulting H_2O_2 is removed by APO in chloroplasts and cytoplasm (Asada, 1992) and by CAT in microbodies (Scandalios, 1987). That CAT is active outside the microbodies is questionable (Nakano and Asada, 1981) because of its low affinity for its substrate (Scandalios et al., 1972). GSH-POX activity, which typically scavenges H_2O_2 in animals, has been detected in tissues and cultured cells of higher plants, but its contribution to H_2O_2 scavenging seems to be negligible (Nakano and Asada, 1981; Drotar et al., 1985). Anyhow, the level of GSH-POX transcripts increases in protoplasts (Criqui et al., 1992; Willekens et al., 1994). Experiments performed using transgenic plants or by specifically inhibiting some scavenging activities indicate that the balance of the overall protective system operating in the cell rather than single enzyme specific activities is crucial for preventing damage induced by oxidative stress (Harper and Harvey, 1978; Klapheck et al., 1990; McKersie et al., 1993; Sen Gupta et al., 1993; Slooten et al., 1995).

Higher plants exhibit other POX activities (guaiacol-POXs) besides APO, and these are involved in cell-wall polymerization, ethylene biosynthesis, and degradation of IAA (Asada, 1992) rather than H_2O_2 scavenging. Siminis et al. (1993) reported a significant difference between recalcitrant and regenerating protoplasts both in guaiacol-dependent POX activity (total POX) and in corresponding IEF zymograms. POX-mediated cell-wall polymerization is H_2O_2 dependent (McDougall, 1992), and different systems have been proposed (Elstner and Heupel, 1976; Angelini and Federico, 1989; Auh and Murphy, 1995) that could ensure H_2O_2 accumulation in the apoplast.

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Abbreviations: APO, ascorbate peroxidase; CAT, catalase; ϵ, extinction coefficient; NBT, nitroblue tetrazolium; POX, peroxidase; SOD, superoxide dismutase.

In this work we have isolated protoplasts from *Nicotiana tabacum* that exhibit both dividing and nondividing potential, according to Siminis et al. (1994). We have followed the changes in the H_2O_2-dependent enzymic activities in the two systems during a 2-week period of protoplast culture and found that (a) a higher scavenging capability could ensure a better regenerating potential and (b) protoplast isolation induces a modification in the activities of the enzymes related to cell-wall reconstitution.

MATERIALS AND METHODS

Plant Material and Isolation of Protoplasts

Tobacco (*Nicotiana tabacum* L. cv Xanthi) plants were grown in a glasshouse at 25°C, and four to five fully developed leaves were collected prior to blooming. Mesophyll protoplasts were isolated and cultured as described (Koop and Schweiger, 1985; Katsirdakis and Roubelakis-Angelakis, 1992b); 4 h of maceration of leaf mesophyll tissue in hydrolytic solution (1% cellulase, 0.5% macerozyme) was used to obtain regenerating protoplasts, and 18 h of maceration in the same hydrolytic solution resulted in recalcitrant protoplasts (Siminis et al., 1994). Unless otherwise specified, the term "regenerating/dividing" is used in the text for protoplasts resulting from the 4 h of maceration and "nonregenerating/nondividing" is used for protoplasts resulting from the 18 h of maceration. Protoplast viability was detected using the Evans blue dye exclusion procedure (Graff and Okong O-Ogola, 1971).

To isolate the membrane fractions, leaf tissue was ground in a mortar and pestle in the presence of 4 volumes of extraction buffer (50 mM K-phosphate, pH 7.5, 20% [w/v] sorbitol, 1 mM ascorbate, 1 mM EDTA, 10 mM DTT, 10 μM leupeptin, 0.3% [v/v] Triton X-100) and filtered through eight layers of cheesecloth, and the homogenate was centrifuged for 20 min at 13,000*g*. The resulting supernatant was further centrifuged for 50 min at 85,000*g* to separate the supernatant from the microsomal pellet, which was resuspended in 50 mM K-phosphate buffer, pH 7.0, 250 mM Suc, 0.5 M KCl, 10% glycerol and then centrifuged under the same conditions twice to wash any unspecifically bound enzymes. The resulting pellet was resuspended in 5 mM K-phosphate buffer, pH 7.8, 250 mM Suc and loaded on a two-phase partition system and the upper, plasma membrane-enriched fraction was extracted for four cycles, as already described (de Marco et al., 1994). Both the first, lower phase and the last, upper phase were collected; these contained the inner membranes, with minor plasma membrane contamination, and right-side out plasma membrane vesicles, respectively (Widell, 1987). They were then stored at −80°C.

Protein Determination and Enzymic Assays

Leaf homogenate was obtained as described above and centrifuged for 15 min at 12,000*g*. The supernatant was collected and either used directly or frozen at −80°C. Lysis of protoplasts was performed by adding 3 volumes of the same extraction buffer and storing the suspension on ice for 30 min with periodic stirring before recovering the supernatant by centrifugation (15 min at 12,000*g*). Protein concentration was quantified according to Lowry et al. (1951). Total protein extracts were used for enzymic assays.

APO activity was determined as described by Nakano and Asada (1981) with minor modifications. One milliliter of the reaction mixture contained 50 mM K-phosphate buffer, pH 7.0, 0.5 mM ascorbate, and 0.2 mM H_2O_2. The reaction was started by adding 10 μg of protein extract. Oxidation of ascorbate was followed at 25°C by the decrease in the A_{290}, using an ϵ of 2.8 mM^{-1} cm^{-1}. One unit of APO was defined as the amount of enzyme that oxidizes 1 μmol of ascorbate per min under the above conditions.

Oxidation of NADH by POX was followed by the decrease in A_{340} at pH 7.0 and 25°C (ϵ: 6.22 mM^{-1} cm^{-1}). One milliliter of the reaction mixture consisted of 50 mM K-phosphate buffer, 0.2 mM H_2O_2, and 0.15 mM NADH. Total POX activity was determined spectrophotometrically at 25°C by measuring the A_{460} (ϵ: 11.3 mM^{-1} cm^{-1}) using 1 mL of a reaction mixture consisting of 50 mM Na-acetate buffer, pH 5.2, 0.5 mM *o*-dianisidine, and 2 mM H_2O_2 (Church and Galston, 1988).

CAT activity was determined by measuring the initial rate of H_2O_2 decomposition at 240 nm (ϵ: 0.036 mM^{-1} cm^{-1}) (Havir and McHale, 1987). One milliliter of the reaction mixture consisted of 50 mM K-phoshate, pH 7.0, and 15 mM H_2O_2. Variations in the H_2O_2 concentration in leaf and protoplast extracts were measured under the same conditions but with the addition of 0.2 mM H_2O_2 to the reaction buffer (for details, see figure legends).

Glutathione-POX was determined according to Klapheck et al. (1990); 1 mL of the reaction mixture contained 50 mM K-phosphate buffer, pH 7.0, 1 mM H_2O_2, 15 mM NADPH, and 0.3 unit of glutathione reductase from bakers' yeast (Sigma). The rate of GSSG formation was determined by its re-reduction as determined by the oxidation of NADPH at 340 nm (ϵ: 6.22 mM^{-1} cm^{-1}).

Units for NADH-POX, total POX, and CAT were defined in analogy with APO units. Apparent V_{max} and H_2O_2-dependent enzymic inhibition were determined from curves of activity obtained by using increasing amounts of the substrate. K_m values were calculated by double-reciprocal plot analysis.

Native IEF, PAGE, and Activity Staining

Native IEF gels to determine total POX and redox activities were run in a mini-gel system (Bio-Rad) as described by Robertson et al. (1987) over a pH range from 3.5 to 11 and from 6 to 11, respectively. POX isoenzymes were visualized by preincubating the native IEF gels for 10 min in 10 mL of methanol and 50 mg of chloro-1-naphthol and successively transferring them for 30 to 60 min to solutions consisting of 50 mM K-phosphate, pH 7.9, and 1 mM H_2O_2 (Lagrimini and Rothstein, 1987). The gels were assayed for redox activity by preincubation in 10% glycerol, 50 mM Na-phosphate buffer, pH 7.4, followed by staining for 30 min at room temperature in the same buffer containing 0.4 mM NADH and 0.5 mM NBT as substrates (Serrano et al., 1994). In some cases, H_2O_2 (10 mM) was added before NBT for comparative detection of NADH-POX activity.

Table I. *Protoplast viability during culture*

Regenerating and nonregenerating protoplasts were cultured for 2 weeks in dark (D) or in light (L) under the conditions described in "Materials and Methods." Values are in percent of initial number of protoplasts.

Protoplasts	Days in Culture				
	0	1	3	6	14
Regenerating D	100	92 ± 6	104 ± 11	Aggregates	Microcalluses
Regenerating L	100	89 ± 7	108 ± 13	Aggregates	Microcalluses
Nonregenerating D	100	91 ± 9	79 ± 9	42 ± 7	11 ± 3
Nonregenerating L	100	66 ± 9	24 ± 6	0	0

Native gels were assayed for SOD activities as described by Laemmli (1970). The activities were visualized by incubating the gels for 45 min in the dark in the presence of 50 mL of 50 mM K-phosphate buffer, pH 7.4, 40 μg of NBT, 4 μg of riboflavin, 100 μL of *N,N,N',N'*-tetramethylethylene diamine, and H_2O_2, as specified in "Results" and then illuminating them until the bands became apparent (Beauchamp and Fridovich, 1971).

RESULTS

Viability of both regenerating and nonregenerating protoplasts was followed during a 2-week culture period (Table I). The number of regenerating protoplasts increased after the 3rd d as a consequence of cell division, and the protoplasts started forming large aggregates. In contrast, the nonregenerating protoplasts decreased throughout the culture period. Although light did not induce any apparent modification in growth of regenerating protoplasts, nonregenerating protoplasts were strongly inhibited (Table I).

The H_2O_2-dependent enzyme activities determined in the donor leaf tissue are presented in Table II. Apparent V_{max} and K_m were estimated using crude extract in order to obtain the approximate range of activity of the different scavenging enzymes. Our data confirmed that CAT and APO have a very different affinity for their substrate, in agreement with results available in the literature (Scandalios et al., 1972; Chen and Asada, 1989). No CAT activity, measured as H_2O_2 loss at 240 nm, was detectable in our extract when H_2O_2 concentrations were below 2 mM (data not shown), whereas APO activity was increasingly inhibited by H_2O_2 concentrations higher than 0.75 mM (18% inhibition 2 min after enzyme addition in the presence of 1 mM H_2O_2; 42% inhibition in the presence of 1.5 mM H_2O_2; complete inhibition using 2.5 mM H_2O_2), indicating that the two H_2O_2-scavenging enzymes operate in slightly overlapping concentrations of H_2O_2. The values of apparent V_{max} and K_m for NADH-POX are reported in Table II. A net production of H_2O_2 in the reaction mixture was found when the change in the H_2O_2 concentration detectable at 240 nm was monitored in the presence of a low initial amount (less than 2 mM) of H_2O_2 (Fig. 1a).

We tried to verify if the increase in the H_2O_2 concentration in the medium containing a low amount of H_2O_2 (0.2 mM) could be the result of a NADH-dependent peroxidative cyclic activity of the kind described by Halliwell (1978). The results reported in Figures 1 and 2 seem to confirm the involvement of the NADH-POX activity in the final H_2O_2 concentration. In fact, in the absence of exogenous NADH both the NADH-eliminating and the H_2O_2-

Table II. *Effect of H_2O_2 concentration on the kinetics of the scavenger enzymes in crude extract from tobacco leaves*

Apparent values of K_m and V_{max} for H_2O_2 and inhibitory concentrations of the substrate for CAT, total POX, APO, NADH-POX, and GSH-POX are shown. SOD activity in presence of H_2O_2 was detected using native gels (see "Materials and Methods"). H_2O_2-induced inhibition was 99% for APO and NADH-POX, and >75% for SODs. ND, Not detectable. –, Not determined.

Enzyme	K_m	V_{max}	Inhibitory [H_2O_2]
CAT	5.3 mM	10.0 ± 1.6 mM	–
Total POX	1.8 mM	4.9 ± 0.7 mM	–
APO	46 μM	330.0 ± 40.0 μM	>2.5 mM
NADH-POX	78 μM	540.0 ± 80.0 μM	>5.0 mM
Cu/ZnSOD	–	–	>250.0 μM
FeSOD	–	–	>75.0 μM
GSH-POX	–	ND	–

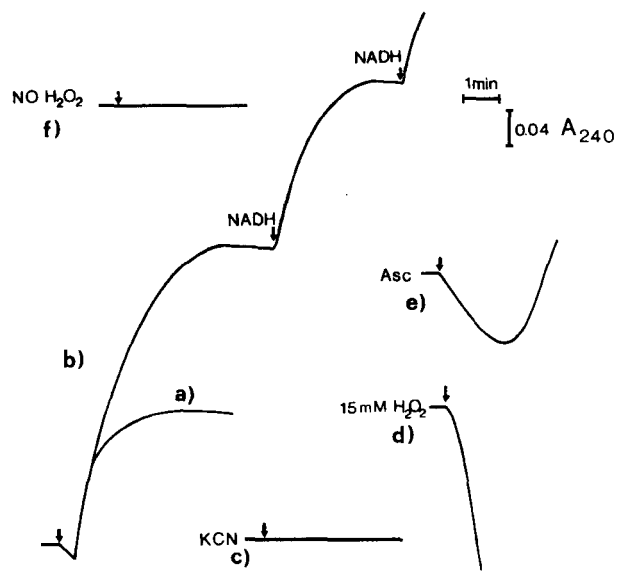

Figure 1. Representative kinetics of H_2O_2 concentration development in crude extract from tobacco leaves. Changes in H_2O_2 concentration were measured as variation in A_{240} in crude extract from tobacco leaves in the presence of 0.2 mM exogenous H_2O_2. a, Reaction started by adding the crude extract (arrows); b, plus 0.15 mM exogenous NADH added to the reaction medium and at each subsequent arrow; c, plus 1 mM KCN; d, plus 15 mM H_2O_2; e, plus 1 mM ascorbate; f, no H_2O_2.

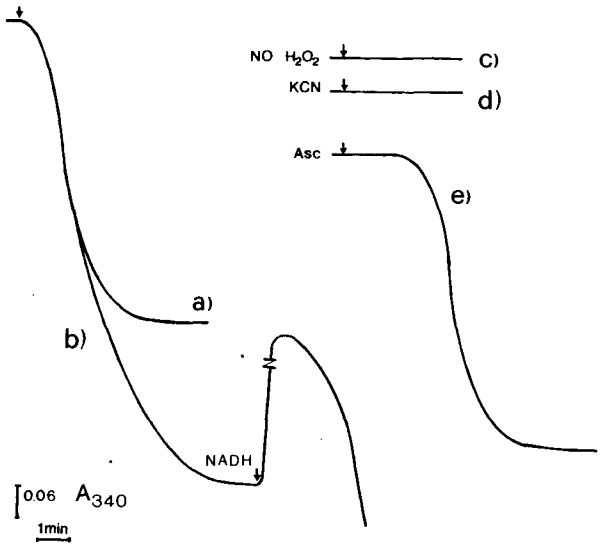

Figure 2. Changes in NADH concentration measured as decrease in A_{340} in crude extract from tobacco leaves in the presence of 0.2 mM exogenous H_2O_2. a, Reaction started by adding the crude extract (arrows); b, plus 0.15 mM exogenous NADH added to the reaction medium and at each subsequent arrow; c, no H_2O_2; d, plus 1 mM KCN; e, plus 1 mM ascorbate.

accumulating reactions started 15 to 30 s after the addition of the extract and stopped after 3 to 4 min (Figs. 1a and 2a); more specifically, during the initial lag phase of the NADH-POX activity, a low H_2O_2 loss was observed and subsequent H_2O_2 accumulation began together with the NADH decrease.

Exogenous NADH, added to the reaction medium before the initiation of the reaction (addition of the extract), stimulated the absolute values of both the oxidized NADH and the accumulated H_2O_2 (Figs. 1b and 2b). The addition of exogenous NADH (0.15 mM) after the termination of the reaction and the corresponding H_2O_2 accumulation induced both NADH oxidation and a further enhancement of H_2O_2 concentration in the reaction medium (Figs. 1b and 2b). When NADH was completely depleted, the H_2O_2 supply slowly decreased, with a rate of about 2% of the V_{max} for CAT (Table II; Fig. 1, a and b).

The reaction was H_2O_2 dependent (Figs. 1f and 2c), and addition of KCN (1 mM), an inhibitor of POX but not of CAT, blocked the H_2O_2 enhancement (Figs. 1c and 2d). Ascorbate (1 mM), which is able to stop the H_2O_2-producing cycle by scavenging the radicals involved in the nonenzymic reactions without inhibiting the H_2O_2-consuming peroxidative reaction, stimulated the H_2O_2 loss until its depletion (Figs. 1e and 2e). The presence of ascorbate prolonged the lag phase before the initiation of the NADH oxidation (Fig. 2e) and, simultaneously with the beginning of the NADH use, the ascorbate-dependent H_2O_2-consuming activity stopped and a net H_2O_2 accumulation was found (Fig. 1e). Moreover, when extracts from protoplasts cultured in light, displaying a higher NADH-POX activity (Table III), were used, both the increase and the decrease in H_2O_2 in the ascorbate-containing reaction medium were more rapid, in a linear manner (data not shown).

When a higher concentration (15 mM) of H_2O_2 was used, a steady loss, independent of NADH concentration, was found (Fig. 1d). The apparent values of V_{max} and K_m of the total POX activity in crude extract from *N. tabacum* leaves are reported in Table II. In the presence of 0.2 mM H_2O_2, the total POX activity was about 10% of the V_{max} (data not shown). Addition of H_2O_2 to the medium resulted in the oxidation of reduced glutathione; such activity, which is H_2O_2 and glutathione reductase dependent, appeared not to be GSH-POX dependent (Table II).

The different SOD isoforms show a wide range of responses with respect to H_2O_2 concentration (Bridges and Salin, 1981). In Figure 3, the activity-staining gels for the H_2O_2-sensitive isoforms of SOD in the presence of increasing amounts of H_2O_2 are shown. Preliminary tests performed using KCN and H_2O_2 (A.P. Papadakis, C.I. Siminis, and K.A. Roubelakis-Angelakis, unpublished data) had indicated that the lower band (b) represents the chloroplastic FeSOD and the upper band (a) is a putative cytoplasmic Cu/ZnSOD. The activity of the FeSOD was already inhibited by 75 μM H_2O_2 (Table II). The Cu/ZnSOD was stimulated by 100 μM H_2O_2 (Fig. 3) and inhibited by 0.25 mM H_2O_2 (Table II); its activity was totally prevented by 0.5 mM H_2O_2 (Fig. 3). The pattern of SOD activity did not basically differ between the regenerating and nonregenerating protoplasts during the first days of culture (A.P. Papadakis, C.I. Siminis, and K.A. Roubelakis-Angelakis, unpublished data).

Total POX activities in regenerating and nonregenerating protoplasts were very similar until the 2nd to 3rd d of culture (Fig. 4A). Later, the regenerating protoplasts showed an enhanced POX activity and started dividing,

Table III. *Light-induced alterations in the enzymic control of H_2O_2 concentration*

Effect of light on H_2O_2-dependent enzymic activities and on the NADH-POX-dependent H_2O_2 production (μmol mg^{-1} protein min^{-1}) in regenerating protoplasts isolated from the mesophyll of *N. tabacum* leaves and cultured for 7 and 14 d in the dark, 7 d in the light, or 6 and 13 d in the dark and then transferred to the light for 1 more d. Enzyme activities were determined as described in "Materials and Methods" and in the legend of Table I and are expressed in units per mg protein as defined in "Materials and Methods."

Enzyme	Days in Dark		Days in Light	Days in Dark + Light	
	7	14	7	6 + 1	13 + 1
CAT	22.0 ± 4.1	19.2 ± 3.2	60.2 ± 10.4	34.4 ± 6.6	72.2 ± 12.0
Total POX	82.4 ± 5.8	114.2 ± 6.4	102.8 ± 9.1	87.3 ± 7.0	110.8 ± 9.2
APO	2.7 ± 0.3	10.1 ± 0.8	8.0 ± 0.8	3.2 ± 0.4	15.7 ± 1.1
NADH-POX	0.9 ± 0.1	4.1 ± 0.5	1.9 ± 0.3	1.4 ± 0.2	5.9 ± 1.1
[H_2O_2]	72.2 ± 8.4	262.3 ± 27.0	168.3 ± 17.0	111.1 ± 13.0	417.4 ± 47.0

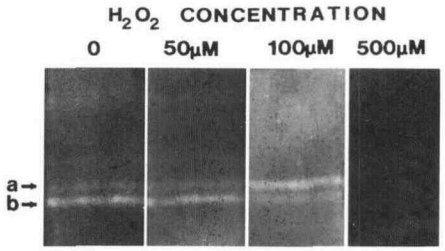

Figure 3. Effect of H_2O_2 concentration on the activity of the H_2O_2-sensitive SOD isoforms. Native gels were stained for SOD activity (200 μg protein/lane) in the presence of increasing concentrations of H_2O_2; a and b indicate the Cu/ZnSOD and FeSOD, respectively.

whereas the nonregenerating ones showed declining POX activity and began to degenerate, as detected by Evans blue staining. After 14 d only about 10% of the nonregenerating protoplasts in culture were still viable (Table I).

The correlation of POX activity with the different POX isoforms on native gels is shown in Figure 4B. Soon after protoplast isolation, the two strongly acidic isoforms specific for leaf tissue (lane L) disappeared (0 d in culture, 4 h of maceration). In dividing protoplasts only an acidic isoform of POX was detectable on the 1st d (lane 1), whereas further bands in the low acidic-neutral and in the basic ranges appeared after the 3rd d (lanes 3 and 6) when total activity exhibited a significant enhancement (Fig. 4A). At the end of the 2nd week, the basic bands became sharper (Fig. 4B, lane 14); no significant difference in the IEF pattern was noticed in protoplasts transferred to light for 1 d after 5 (lane 5+1) and 13 (lane 13+1) d in the dark, when compared to protoplasts of the same age kept in the dark (lanes 6 and 14, respectively). No band for POX activity was detectable after 18 h of maceration (lane 0, 18h maceration), whereas the POX zymogram of nondividing protoplasts indicated that both the acidic and the basic bands were already present on d 2 (lane 2); at d 6, the isoforms of POXs disappeared (lane 6). There was good correlation between activities determined spectrophotometrically (Fig. 4A) and those visualized in the IEF (Fig. 4B).

The changes of APO activities in cultured protoplasts (Fig. 5A) had a pattern similar to that of the total POX activities, but in contrast, APO activity was strongly enhanced by light (Table III). Addition of ascorbate (1 mM) to the reaction medium stimulated total POX activity in the range of H_2O_2 concentrations suitable for APO assays (data not shown). Two isoforms of APO have been described, one cytoplasmic and the other chloroplastic; the latter is extremely labile in the absence of its substrate (Nakano and Asada, 1987; Chen and Asada, 1989). In a preliminary assay, we extracted APO from leaves using either a detergent-containing or a detergent-free buffer to roughly estimate the ratio of the two isoforms. Since 65% of total activity was detergent dependent and we did not succeed in either isolating or purifying APO in the absence of its substrate, we suggest that most of the leaf activity is chloroplastic. No ascorbate oxidation was detectable in a H_2O_2-depleted medium, indicating the absence of ascorbate oxidase (data not shown).

The NADH-POX activity was also monitored during a 2-week culture period. In nondividing protoplasts, the activity was almost undetectable, whereas in dividing protoplasts, although it was very low for the first days, it steadily increased with time (Fig. 5B). Light slightly stimulated the NADH-POX activity (Table III). In leaf extracts, NADH-POX activity resulted in the accumulation of H_2O_2 in the medium (cf. Figs. 1 and 2). Similarly, a strong correlation between the increasing NADH-POX activities and H_2O_2 concentrations in the reaction medium was found using dividing protoplasts (cf. Figs. 5B and 6). Nondividing protoplasts showed no NADH-POX activity (Fig. 5B), and no corresponding H_2O_2 accumulation was detectable (Fig. 6). When samples still exhibiting CAT activity (Fig. 5C, 0–3 d in culture) were used, H_2O_2 was removed from the medium (Fig. 6, 0–3 d in culture) and remained constant when the samples used had no CAT activity (cf. Figs. 6 and 5C, 6–14 d in culture). Native gels stained for redox activity in the presence and absence of H_2O_2 did not give reproducible results (data not shown). Also, an attempt was made to localize the NADH-POX activity in tobacco leaf cells. Data in Table IV show that such activity seems to be

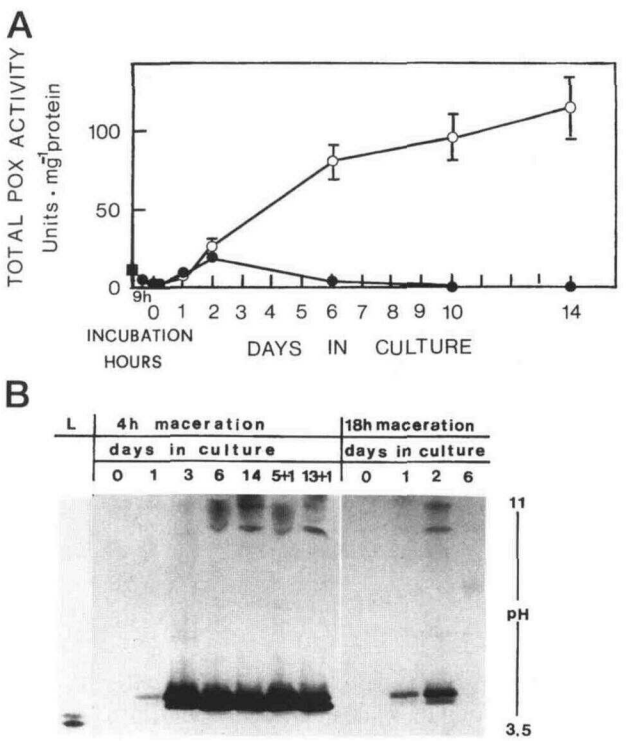

Figure 4. Total POX activity and comparison of POX isoforms during the maceration period and the subsequent culture of regenerating and nonregenerating tobacco protoplasts. A, Time course of total POX activity (units/mg protein ± SE) during the maceration period and the subsequent culture of regenerating (○) and nonregenerating (●) tobacco protoplasts. B, IEF gels (pH 4–11) stained for total POX activity (10 μg protein/lane) using extracts from leaves (lane 1); from protoplasts isolated after 4 h of maceration and cultured in the dark for 0, 1, 3, 6, or 14 d (lanes 2–6) or cultured for 5 and 13 d in the dark plus 1 d in the light (lanes 7 and 8); and from protoplasts isolated after 9 h of maceration (lane 9) or after 18 h of maceration and cultured in the dark for 1, 2, or 6 d (lanes 10–12).

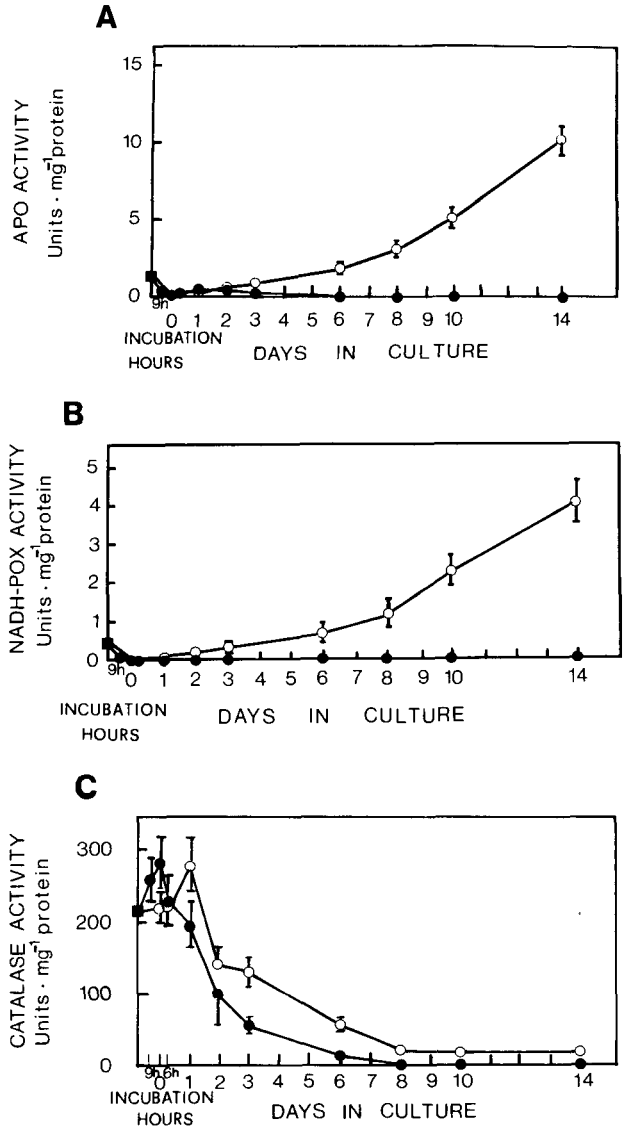

Figure 5. Time course of APO (A), NADH-POX (B), and CAT (C) activities (units/mg protein ± SE) during the maceration period and the subsequent culture of dividing and nondividing tobacco protoplasts isolated after 4 (dividing, ○) and 18 h (nondividing, ●) of incubation with maceration enzymes.

present both bound to plasma membrane vesicles and in 85,000*g* supernatant. The membrane-bound activity was about 8% of the activity recovered in the supernatant, was external, since it was measured using right-side-out vesicles in the absence of detergent, and was strictly dependent on the POX stimulators $MnCl_2$ and salicylhydroxamic acid (Halliwell, 1978) (data not shown).

Results showing CAT activity (Fig. 5C) basically confirmed our previously reported data for the first 7 d of culture (Siminis et al., 1994). In this work the period of protoplast culture was extended to 14 d. The pattern of CAT activity did not differ between dividing and nondividing protoplasts, although the absolute values were higher in regenerating protoplasts during culture. The activity staining of native gels (data not shown) confirmed the results already published (Siminis et al., 1994). Light exhibited a stimulatory effect on CAT in regenerating protoplasts (Table III).

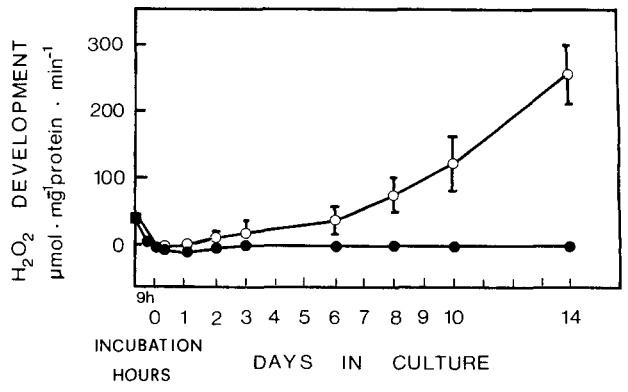

Figure 6. Development of the H_2O_2 concentration during the maceration period and the subsequent culture of dividing and nondividing tobacco protoplasts isolated after 4 (dividing, ○) and 18 h (nondividing, ●) of incubation with maceration enzymes; 0.2 mM NADH and 0.2 mM exogenous H_2O_2 were added to the reaction buffer.

DISCUSSION

The isolation of protoplasts from leaf mesophyll results in an alteration of cell metabolism; an increase of toxic forms of oxygen has been detected, which has been suggested to be the cause of poor protoplast regeneration (Roubelakis-Angelakis, 1993). Toxic oxygen stimulates lipid peroxidation in cultured tissues (Benson and Roubelakis-Angelakis, 1993, 1994) and directly inhibits many enzymes; for instance, 10 μM H_2O_2 is inhibitory for some enzymes involved in the photosynthetic CO_2 fixation (Kaiser, 1976) and 50 μM H_2O_2 is inhibitory for the cytoplasmic enzyme aconitase (Verniquet et al., 1991). A total H_2O_2 content of 138 μmol kg^{-1} fresh weight has been reported for germinating endosperm (Warm and Laties, 1982), but a method for directly and precisely detecting the H_2O_2 concentration in the different cell compartments in vivo has not yet been developed and the critical concentration of toxic oxygen has not been defined for all of the cell components. What seems to be evident is that oxidative stress is compartment specific. It has been observed that

Table IV. *Distribution of NADH-POX activity among the cell fractions isolated from tobacco leaves*

The fractions examined are the crude filtrate (1), supernatant (2), and pellet (3) recovered after the 13,000*g* centrifugation; the supernatant (4) and microsomal fraction (5) after salt washing and 80,000*g* centrifugation; the inner membrane fraction (6); and the plasma membrane (7) fraction. ND, Not detectable.

NADH-POX	Cell Fraction						
	1	2	3	4	5	6	7
Specific activity (units mg^{-1} protein)	0.45	0.61	ND	0.54	1.25	ND	3.15
Total activity (units)	12.1	12.4	0	11.1	1.0	0	0.8

stress factors that lead to organ-specific accumulation of toxic forms of oxygen simultaneously stimulate the transcription of the scavenging isoenzymes operating in the respective compartments (Willekens et al., 1994).

We observed that the H_2O_2 concentration present in the reaction buffer could definitely modify the activities of the scavenging enzymes and therefore the dynamic of its concentration (Table II) and attempted to estimate its in vivo physiological concentration. We assumed that it should be in the range that ensures APO activity (Table II), since the role of this enzyme is just to regulate the concentration of its substrate in chloroplasts (Nakano and Asada, 1981) and in the cytoplasm (Klapheck et al., 1990) and it could not operate in the presence of H_2O_2 concentrations higher than 0.75 mM because of the substrate-induced inhibition. More specifically, we found that 0.075 and 0.25 mM H_2O_2 were already inhibitory for the chloroplastic and the cytoplasmic SODs, respectively (Table II), and therefore 0.2 mM H_2O_2 was used when putative cytoplasmic H_2O_2-dependent activities were measured using crude extract. Higher H_2O_2 concentrations, incompatible with the activity of many chloroplastic and cytoplasmic enzymes, may occur in vivo in the microbodies or in the apoplast, but in these cell compartments the potential danger of H_2O_2 is strongly limited by the high activity of H_2O_2-exploiting enzymes (CAT and guaiacol-POX), which can rapidly remove their substrate (Table II). We could not measure any GSH-POX activity, confirming its negligible, if any, contribution to scavenging H_2O_2 in higher plants (Klapheck et al., 1990; Cakmak et al., 1993).

Our data indicate that isolation-induced oxidative stress may contribute to preventing protoplast regeneration, since only protoplasts expressing high H_2O_2-scavenging activities were able to survive and divide (Fig. 5, A and C). The precise intracellular localization of the accumulation of the toxic active forms of oxygen during protoplast isolation is still unknown. Lipid peroxidation activity was enhanced in protoplasts in which the APO pathway for H_2O_2 scavenging was impaired (May and Leaver, 1993), and regenerating protoplasts showed increased APO activity and a decline in CAT activity after a transient increase (Fig. 5, A and C). Since they were grown in the dark, we suggest that the cytoplasmic isoform of APO is responsible for the enhanced scavenging activity.

It has been shown that protoplasts cannot divide unless cell-wall reconstitution is accomplished (Hahne and Hoffmann, 1984; Katsirdakis and Roubelakis-Angelakis, 1992a), and our results confirm that total POX activity, which is considered to be indicative of the cell-wall polymerization rate (Gaspar et al., 1985), was significantly higher in dividing than in nondividing protoplasts during culture (Fig. 4, A and B). Furthermore, NADH-POX activity, which has been proposed to generate H_2O_2 (Elstner and Heupel, 1976; Gross et al., 1977), was not detectable at all in nondividing protoplasts (Fig. 5B), which were not able to produce H_2O_2 (Fig. 6). These data show that on the one hand, oxidative stress may reduce the regeneration potential of protoplasts, and on the other hand, only protoplasts that are able to supply H_2O_2 can actually divide. Furthermore, the recalcitrant (nondividing) protoplasts do not exhibit either sufficient toxic H_2O_2-scavenging activity (Fig. 5, A and C) or sufficient H_2O_2-accumulating activity to use in cell wall reconstitution (Figs. 5B and 6); the death of protoplasts is accelerated if oxidative stress is enhanced by exposure to light (Table I) and they have a significantly lower total POX activity (Fig. 4). It is also worth noting that dividing protoplasts do not undergo lipid peroxidation during culture, whereas the recalcitrant protoplasts are prone to such damage (C.I. Siminis, A.P. Papadakis, and K.A. Roubelakis-Angelakis, unpublished data), even though they do not have an enzymic H_2O_2-generating system.

We suggest a different intracellular localization of scavenging and H_2O_2-producing enzymes. In fact, the two described forms of APO are chloroplastic and cytoplasmic (Asada, 1992); three different SOD isoenzymes are localized in mitochondria, chloroplasts, and cytoplasm (Bannister et al., 1987); and CAT isoforms have been found in microbodies and mitochondria (Scandalios et al., 1980). In contrast, a H_2O_2-generating NADH-POX activity has been described to be bound to the cell wall (Goldberg et al., 1985). Also, an outer plasma membrane-linked NAD(P)H-oxidase activity able to induce H_2O_2 accumulation has been detected using plasma membrane vesicles (Askerlund et al., 1987). Furthermore, the apoplastic localization of the POXs involved in cell-wall polymerization has been widely demonstrated (Church and Galston, 1988; MacAdam et al., 1992; McDougall, 1992). Toxic oxygen-scavenging enzymes show a highly varying affinity for their substrate (Table II; Fig. 3), and this further confirms the existence of different thresholds in the different cell compartments. Our results and the information available in the literature (Kaiser, 1976; Verniquet et al., 1991) support a model for which low concentrations of H_2O_2 are highly toxic in chloroplasts and cytoplasm, whereas H_2O_2 becomes a necessary substrate in the apoplast in order to ensure the cross-linkages of cell-wall components (McDougall, 1992). Using crude leaf extract, we measured NADH-POX activity using both right-side out plasma membrane vesicles and the 85,000*g* supernatant (Table IV). Although the simultaneous presence of both the cytoplasmic and the apoplastic fractions does not rule out a vacuolar contribution to the NADH-POX activity, the apoplastic localization of at least the plasma membrane-bound activity seems probable.

The results of the experiment in which the regenerating protoplasts were cultured in the light (Table III) support the hypothesis of the existence of two independent systems regulating the H_2O_2 concentration in different cell compartments. The cell-wall polymerization-related activities (total and NADH-POX activity) were only slightly affected by light, whereas the scavenging activities of CAT and APO were strongly stimulated. This could indicate that regenerating protoplasts are able to discriminate between toxic H_2O_2 that is produced during photosynthetic metabolism inside the cell and H_2O_2 that is produced for ensuring cell-wall reconstitution and is presumably apoplastic. Furthermore, the data suggest that the oxidative stress induced during protoplast isolation has a very peculiar character. In fact, different stress factors such as ozone, UV,

and cold are able to stimulate the scavenging-enzyme activities in tolerant plants, and the enhanced protection against oxidative stress induced by only one of these factors is sufficient to control the added oxidative effect of more stress factors, a phenomenon known as cross-tolerance (Van Camp et al., 1994). In contrast, in cultured regenerating protoplasts, light induced a further increase in the already stimulated scavenging activities (Table III).

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